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The mechanisms by which moderate hypothermia (32°C for 12-72 h) affect human cellular function are unclear. We tested the hypothesis that it produces broad changes in mRNA expression in vitro. Acute monocytic leukemia (THP-1) cells were incubated under control conditions (37°C) or moderate hypothermia (32°C) for 24 h. RNA was extracted, and the hypothermic response was confirmed by examining the expression of the cold-induced RNA-binding protein (CIRBP) gene by RT-PCR. Gene expression analysis was performed on seven sets of paired samples with Affymetrix U133A chips using established statistical methods. Sequences were considered affected by cold if they showed statistically significant changes in expression and also met published post hoc filter criteria (changes in geometric mean expression of _2-fold and expression calls of "present" or "marginal" in at least half of the experiments). Changes in the expression of selected sequences were further confirmed by PCR. Sixty-seven sequences met the criteria for increased expression (including cold-inducible genes CIRBP and RNA binding motif 3), and 100 sequences showed decreased expression as a result of hypothermia. Functional categories affected by hypothermia included genes involved in immune responses; cell growth, proliferation, and differentiation; and metabolism and biosynthesis. Several heat shock proteins (HSPs) showed decreases in expression. Moderate hypothermia produces substantial changes in gene expression, in categories potentially of systemic importance. Cold exposure without rewarming decreased the expression of several HSPs. These in vitro findings suggest that prolonged hypothermia in vivo might be capable of producing physiologically relevant changes in gene expression by circulating leukocytes

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Effect of moderate hypothermia on gene expression by THP-1 cells: a DNA microarray study

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cold stress; thermal stress; heat shock; mononuclear cells

HUMANS RESPOND TO ENVIRONMENTAL COLD with behavioral changes, such as putting on a coat, taking shelter, and moving closer to a heat source, and physiological alterations, such as shivering and cutaneous vasoconstriction (2). Although highly effective, these systemic adaptations can be overwhelmed or circumvented under conditions such as severe environmental exposure, general anesthesia, trauma, and some cases of sepsis, and, indeed, the induction of hypothermia during some pathophysiological states can lead to significant adverse side effects (15). Mammals are also affected by hypothermia at the cellular level. These cellular effects of hypothermia include decreases in oxygen consumption and metabolic rate, alterations in redox state (16), and gene expression program (15, 19). Some of the genes shown to be directly affected by hypothermia include cold-induced RNA-binding protein (CIRBP) (13, 14) and RNA binding motif 3 (RBM3) (4). Others, observed after hypothermic exposure in vivo, are likely the result of the compensatory activation of the sympathetic nervous system that occurs in response to hypothermia (2). Still others, such as TNF-α and IL-1β, have been demonstrated in vitro in the context of a concomitant underlying stimulus such as LPS (5). It thus appears that cold can alter gene expression through direct effects on cells, through cellular responses to systemic signals, and through modulation of the effects of other stimuli. However, the full extent to which hypothermia alters gene expression in human cells is unknown.

Although hypothermia can occur in and complicate pathological states, moderate systemic hypothermia has also been found to be of therapeutic value in select clinical settings. Mild to moderate systemic hypothermia (32–34°C for 12–24 h) has been found to improve neurological outcomes after out-of-hospital cardiac arrest (1, 9) and may be beneficial in selected patients with severe closed head injury (11, 12). Recently, systemic hypothermia of 33.5°C for 72 h was found to improve both survival and neurological outcomes in newborn infants suffering from hypoxic-ischemic encephalopathy (17). Although the precise cellular mechanisms leading to the benefits are unclear (16), it is noteworthy that the degrees of induced hypothermia in these studies were comparable with what is required to induce changes in gene expression in mammalian cells in vitro. This raises the question of whether or not some of the beneficial effects of therapeutic hypothermia might be attributable to changes in gene expression, either in inflammatory cells or in tissues themselves.

The acute mononcytic leukemia cell line THP-1 is an established in vitro system for analyzing the effects of hypothermia on cytokine secretion and gene expression in response to proinflammatory stimuli such as LPS (6). Moderate hypothermia (32°C) alters the kinetic profile of LPS-stimulated expression of TNF-α and IL-1β (6), attributable to the augmentation of NF-κB activation through a cold-induced delay in the reexpression of IκB-α after LPS stimulation (5). THP-1 cells are thus clearly capable of responding to hypothermia in the presence of a proinflammatory stimulus. However, it is not

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known to what extent hypothermia can alter gene expression in the absence of a proinflammatory stimulus. A better knowledge of which genes are affected by hypothermia in models of inflammatory cells (e.g., THP-1) is required if we are to identify additional mechanisms that may be susceptible to manipulation for therapeutic benefit.

In this study, we report on the use of DNA microarrays to test the hypothesis that exposure to moderate hypothermia, in the range known to produce clinically beneficial effects in vivo (32°C for 24 h), is sufficient to induce extensive changes in gene expression in THP-1 cells in vitro.

MATERIALS AND METHODS

Cell culture and hypothermic exposure. The acute monocytic leukemia cell line THP-1 was obtained from the American Type Culture Collection and maintained in culture using RPMI 1640 medium with standard additives in the presence of 10% defined FBS certified to have <0.01 EU/ml endotoxin activity (Hyclone; Logan, UT). Cultures were maintained in 5% CO_2 tissue culture incubators maintained at 37°C until ready for use. Cells were grown to ~80% confluence before the hypothermic exposure.

The hypothermic exposure was delivered by placing a flask of cells into a 5% CO_2 incubator maintained at a temperature of 32°C for 24 h. Control cells were maintained at 37°C. Both exposures were allowed to proceed in parallel; thus each individual experiment consisted of a paired set of flasks. With one exception, all of the experiments reported here were run on separate days; in the sole exception (which occurred in the samples used for microarray analysis), one hypothermic exposure was started in the morning and another in the afternoon, leading to some overlap of the time periods in which the two sets of paired samples were in the incubators.

RNA isolation. Total RNA was isolated from cells using RNeasy mini-columns (Qiagen) following the instructions of the manufacturer. The quality of RNA was assessed using standard techniques (7), including examination of the absorption spectrum in the range 230–320 nm, measurement of the 260-to-280-nm optical density absorbance ratio, and by detection of clear 28S and 18S rRNA bands on ethidium bromide-stained agarose gels.

A sample pair was considered acceptable for microarray analysis if the quality of the RNA was suitable and if an increase in the expression of the CIRBP gene was detected in the 32°C sample by RT-PCR, as illustrated in Fig. 1. Of the 13 experiments performed (8 for the purpose of microarray analysis and 5 for the subsequent confirmatory PCR), 12 experiments met the CIRBP expression criterion. The CIRBP responses of the seven samples included in the microarray analysis are illustrated in Fig. 1. The rejected experiment (intended for microarray analysis) demonstrated high levels of CIRBP expression in the controls that did not increase further in cells exposed to moderate hypothermia. All five samples prepared for confirmatory PCR of the microarray findings demonstrated increases in CIRBP in response to cold stress; of these, the three that best illustrated the responses of other genes to moderate hypothermia were chosen for presentation here (see RESULTS).

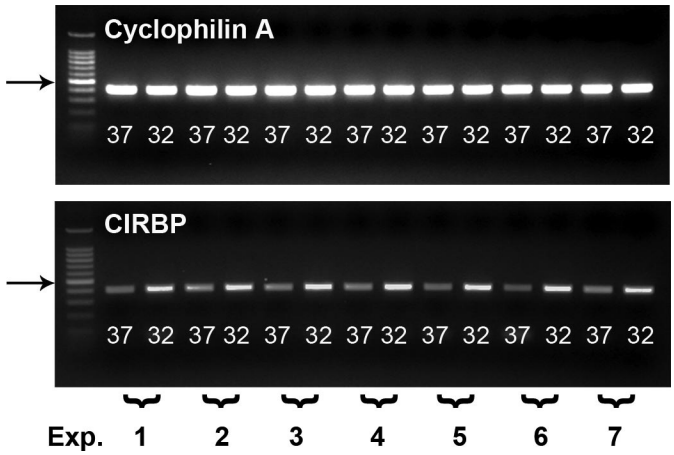

Fig. 1. RT-PCR demonstrating the effect of moderate hypothermia on the expression of cold-induced RNA-binding protein (CIRBP) and cyclophilin A. All of the paired samples sent for microarray analysis are represented. The ladder bands (*far left*, unlabeled lane) occur in 100-bp increments; arrows point to the bright 500-bp bands in the ladders. Exp, experiment.

Microarray hybridization. Gene expression analysis was performed using Affymetrix U133A arrays (version 1). Hybridization to the array was performed by the Partners Gene Chip Array Technology Center (Cambridge, MA) following the manufacturer's instructions, as described in detail elsewhere (20). Because of a switch in equipment used by our core laboratory, scanning of the first four paired sets of samples was performed using model 2500 Affymetrix scanner and preprocessed with CGOS 1.2 (first 4 experiments); the last three paired sets of samples were scanned with an model 3000 Affymetrix scanner and preprocessed with CGOS software. Mean clipped signal intensities were set to 100.

Expression analysis. For each sequence on the U133A array, a cold-to-control expression ratio was obtained by dividing the signal obtained from cells exposed to 32°C by the signal obtained from cells exposed to 37°C. To prevent expression ratios from becoming infinite or incalculable in log space, any expression signals of zero were reset to 0.01; this was chosen because the next-lowest signal intensity possible on the chips was 0.1. Only 12 sequences on the chip required this adjustment.

Sequences significantly affected by hypothermia were identified using previously established methods (18, 20, 22). Briefly, the expression ratios were log transformed (natural log), and, for each sequence, the mean and 95% confidence intervals (CI) on the mean were computed using the *T*-distribution. Expression ratios with 95% CI that excluded zero [i.e., ln(1-fold) = 0] were considered to have a statistically significant difference in expression between cold and control cells. Although computations for statistical significance were performed on natural log-transformed data, for ease of interpretation, means and CI were transformed back to base 10 for the purposes of reporting. Accordingly, the means presented in this study represent geometric means and are not located in the arithmetic center of the reported base 10 CI.

To reduce false-positive reports, where stated in the study, post hoc filtering of the data was performed as follows. First, sequences were required to display a twofold or greater change in expression. Second, the presence/absence calls reported by CGOS 1.2 software were required to be "present" or "marginal" in at least half (in this case, 4 of 7 experiments) of the hypothermic cells (for upregulated sequences) or in the control cells (for downregulated sequences). This general approach to post hoc filtering has been used in previous work, with slight variations (18, 20, 22), and has been found to yield results that are both congruent with published literature and replicable by RT-PCR.

The CGOS 1.2 output files have been uploaded into the National Center for Biotechnology Information (NCBI) gene expression omnibus database under series Accession No. GSE4425.

Confirmatory RT-PCR. Poly-T primed RT-PCR was performed using the Retroscript First-Strand Synthesis Kit (Ambion; Austin, TX) following the manufacturer's instructions. Four micrograms of RNA per sample were used for each reaction. After the RT, samples were diluted to a concentration of 50 ng/μl and further amplified by conventional PCR as described previously (18, 20) using *Taq* polymerase and buffer obtained from Promega (Madison, WI). The primer sequences used in these reactions are shown in Table 1.

Table 1. *Primer sequences used in this study*

Primer Set	GenBank Accession No.	Sequences		Melting Temperature, °C	Amplicon Size, bp
		Sense	Antisense		
Cyclophilin A	BC018843	5′-AGGTCCCAAAGACAGCAGAA-3′	5′-TGTCCACAGTCAGCAATGGT-3′	60	406
CIRBP	D78134	5′-CTGCTCAAGATCGTCCTTCC-3′	5′-GGATTCACAGTCCGTTCGAT-3′	60	401
LSP-1	NM_002339	5′-CCGTCCTGCAAGGATATTGT-3′	5′-AAGGGCAGAGAGGCTAAAGG-3′	60	381
MAFF	NM_012323	5′-TGGCAAATAGGGAGACAAGG-3′	5′-CTGCCTTCTGGGCTTAAGTG-3′	60	586
IGF-1	AI972496	5′-TGGTATTTGGGGCCTTTATG-3′	5′-TATTTGCCCAAAATGCACTG-3′	60	314
C-type lectin-1	AF313468	5′-GGGCTCTCAAGAACAATGGA-3′	5′-GGTACCCAGGACCACAGCTA-3′	60	213
CD14	NM_000591	5′-GGTTCCTGCTCAGCTACTGG-3′	5′-CTTGGCTGGCAGTCCTTTAG-3′	60	595
TNF-α	NM_000594	5′-AGCCCATGTTGTAGCAAACC-3′	5′-GGTTGAGGGTGTCTGAAGGA-3′	60	516
HSP105	BG403660	5′-ATGCTGCTCCTTTCTCCAAA-3′	5′-CAGTTTGTACCTGGGGCTGT-3′	60	368
HSP47	NM_004353	5′-AGCAGCAAGCAGCACTACAA-3′	5′-TTCCCCATCCAGATCTTCAG-3′	60	443
GAPDH	Commercial*	5′-CCACCCATGGCAAATTCCATGGCA-3′	5′-TCTAGACGGCAGGTCAGGTCCACC-3′	60	600

CIRBP, cold-induced RNA-binding protein; LSP-1, lymphocyte-specific protein-1; MAFF, v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family protein F; HSP, heat shock protein. *Obtained from Stratagene.

RESULTS

Number of affected sequences. Of the 22,283 sequences on the Affymetrix U133A chip, 167 sequences met our prospectively defined criteria as being affected by moderate hypothermia. Of these, 100 sequences were decreased by cold exposure and 67 sequences were increased. These values were derived as follows: 4,832 sequences (2,631 decreased and 2,201 increased) showed statistically significant changes in expression as defined by 95% CI on the geometric means that excluded onefold [= ln(0)]. Of these 4,832 sequences, 516 sequences (320 decreased and 196 increased) showed absolute changes in expression of twofold or greater. However, only about one-third of these sequences (167 sequences) also had expression calls of "present" or "marginal" in a majority of experiments under control conditions (for decreased sequences) or in the

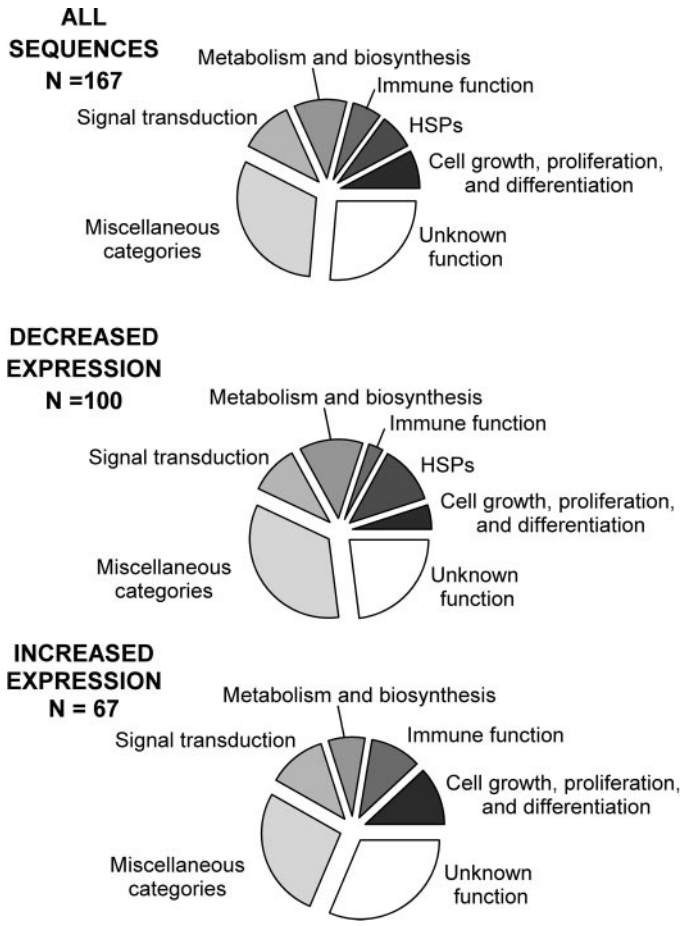

Fig. 2. Principal functional classes affected by moderate hypothermia. The HSP category includes heat shock proteins, chaperonins, and cochaperonins. Note that no HSPs were present in the final list of upregulated genes (*bottom*).

Table 2. *Effect of moderate hypothermia on the expression of important control (housekeeping) sequences*

Common Name	GenBank Identifier	Cold-to-Control Expression Ratio
Cyclophilin A sequences		
Cyclophilin A, T cell	BE731738	0.92 (0.83–1.01)
Cyclophilin A	AI191118	1.09 (0.32–3.64)
Cyclophilin A	AI708767	0.91 (0.84–0.98)*
Cyclophilin A	NM_021130	0.92 (0.84–1.00)*
Cyclophilin A	BC005982	0.91 (0.84–0.98)*
Cyclophilin A	BC018843	0.92 (0.85–0.99)*
GAPDH		
5′-Sequence	M33197	1.03 (0.96–1.10)
Middle sequence	M33197	1.01 (0.93–1.10)
3′-Sequence	M33197	1.03 (0.94–1.12)
β-Actin		
5′-Sequence	X00351	0.97 (0.90–1.05)
Middle sequence	X00351	0.97 (0.85–1.10)
3′-Sequence	X00351	0.97 (0.85–1.10)
Ribosomal proteins		
Ribosomal protein S2	NM_002952	0.93 (0.81–1.05)
Ribosomal protein S2	AI183766	0.92 (0.79–1.08)
Ribosomal protein S3	U14990	0.98 (0.88–1.09)
Ribosomal protein S3A	NM_001006	0.91 (0.76–1.10)
Ribosomal protein S10	NM_001014	0.92 (0.83–1.02)
Ribosomal protein S12	AI799007	0.93 (0.85–1.03)
Ribosomal protein S20	NM_001023	0.97 (0.87–1.07)
Ribosomal protein S23	NM_001025	0.93 (0.83–1.05)
Ribosomal protein S29	NM_001032	0.96 (0.87–1.06)
Ribosomal protein L13a	BC001675	0.93 (0.83–1.04)
Ribosomal protein L13a	BF942308	0.96 (0.85–1.07)
Ribosomal protein L30	L05095	0.91 (0.81–1.03)
Ribosomal protein L37a	NM_000998	0.94 (0.85–1.05)
Ribosomal protein L38	AW303136	0.93 (0.68–1.28)
Ribosomal protein L41	NM_021104	0.93 (0.82–1.06)
Large Ribosomal protein P1	NM_001003	0.91 (0.81–1.03)
Alu-Sq subfamily consensus sequence	U14573	0.94 (0.82–1.08)

Expression ratios are shown as geometric means, with the 95% confidence intervals in parentheses. *Statistically significant change ($P \leq 0.05$).

cold-exposed cells (for increased sequences). It is noteworthy that the number of sequences that were decreased by cold exceeded the number of sequences increased by this stress at all steps of post hoc filtering.

Functional categories of affected sequences. Figure 2 illustrates the distribution of functional categories to which the affected sequences were assigned. The classification was performed manually and was based on resources available at the NCBI Web site (**http://www.ncbi.nlm.nih.gov**), including Entrez Gene and Online Mendelian Inheritance in Man, and it mirrors previous work in our laboratory (18, 20–22). It should be noted that a number of sequences corresponded to genes that could reasonably be put into more than one of the functional classes; however, only one principal functional category was assigned to each sequence.

The functional categories that included the largest number of sequences affected by cold stress were as follows: unknown, 44 sequences (23 decreased and 21 increased); signal transduction including MAPK pathway elements, 18 sequences (10 decreased and 8 increased); metabolism and biosynthesis, 18 sequences (13 decreased and 5 increased); immune function, 10 sequences (3 decreased and 7 increased); heat shock proteins (HSPs) including chaperonins and cochaperonins, 12 sequences (all decreased); and cell growth, proliferation, and differentiation, 13 sequences (5 decreased and 8 increased).

Effect of moderate hypothermia on the expression of control sequences. The effects of moderate hypothermia on control sequences are shown in Table 2. Table 2 lists sequences that showed little to no change in expression in a broad number of contexts (18, 20–22) as well as a number of sequences (such as GAPDH and β-actin) that are widely used as a reference point for gene expression data. Of the 18 genes presented in Table 2, only one (cyclophilin A) was represented by sequences that showed a statistically significant change in expression as a result of moderate hypothermia and all (including the sequences that did have a statistically significant change in expression) had geometric mean expression ratios that fell within 10% of onefold.

THP-1 cells have been found to differentiate in culture in response to a number of stimuli other than hypothermia (3, 8). To ascertain whether there was evidence of hypothermia-induced differentiation under our experimental conditions, we examined the expression of sequences corresponding to antigens CD68, CD36, and CD11b (8). Five sequences were identified on the array corresponding to these genes: one corresponding to CD68 and two each corresponding to CD36 and CD11b. Hypothermia had no effect on the expression of four of these sequences [geometric mean expression ratios (with 95% CI in parentheses): CD68, 0.81 (0.54–1.2); CD36, 0.95 (0.74–1.2) and 1.2 (0.40–3.9); and CD11b, 0.89 (0.65–1.2)]. The fifth sequence, corresponding to CD11b, showed a statistically significant change in expression that was well below our cutoff level of twofold [geometric mean expression ratio of 1.4 (95% CI: 1.2–1.7)].

Sequences affected by moderate hypothermia. Tables 3 and 4 show the sequences most strongly affected by moderate hypothermia. Included are all sequences that met our post hoc filter criteria and showed changes in expression of ≥2.5-fold (or ≤0.40-fold in the case of decreased sequences).

Table 3. *Sequences most strongly decreased by moderate hypothermia*

Functional Category	Common Name(s)	GenBank Identifier	Cold-to-Control Expression Ratio
Cell growth, proliferation, and differentiation	TLE1; transducin-like enhancer of split [E(sp1) homolog, *Drosophila*]	NM_005077	0.34 (0.16–0.73)
	TUSC4; tumor suppressor candidate 4; homologous to yeast nitrogen permease (candidate tumor suppressor)	NM_006545	0.39 (0.21–0.73)
Hormones and neurotransmitters	PAM; peptidylglycine alpha-amidating monoxygenase	NM_000919	0.35 (0.20–0.62)
HSPs, chaperonins, and co-chaperonins	SERPINH1; HSP 47; colligin1; colligin2; SERPINH2	NM_004353	0.17 (0.12–0.25)
	HSPH1; heat shock 105kDa/110kDa protein 1; HSP 105	BG403660	0.28 (0.20–0.41)
		NM_006644	0.29 (0.25–0.33)
	APG-1; heat shock protein (hsp110 family); HSP 110	NM_014278	0.34 (0.22–0.53)
	HSPA1A; heat shock 70kDa protein 1A; HSP 70-1	NM_005345	0.36 (0.25–0.50)
		NM_005345	0.37 (0.26–0.53)
Immune function	BCL11A; B-cell CLL/lymphoma 11A (zinc finger protein)	NM_022893	0.36 (0.29–0.45)
Membrane protein	ITM1; integral membrane protein 1; transmembrane conserved gene; TMC	L38961	0.38 (0.28–0.50)
Membrane transport	ATP6V1D; ATPase, H^+ transporting, lysosomal 34kDa, V1 subunit D	AF077614	0.38 (0.31–0.47)
Metabolism and biosynthesis	HMGCR; 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HMG-CoA reductase	NM_000859	0.37 (0.31–0.43)
	PIGF; phosphatidylinositol glycan, class F	NM_002643	0.39 (0.33–0.47)
	SC4MOL; sterol-C4-methyl oxidase-like	NM_006745	0.40 (0.37–0.43)
RNA processing	RBM10; RNA binding protein motif 10	AW409974	0.26 (0.18–0.39)
		AL137421	0.37 (0.27–0.50)
RNA stability and degradation	DKFZP566E144; small fragment nuclease; CGI-114	NM_015523	0.40 (0.32–0.49)
Signal transduction	CNR1; cannabinoid receptor 1 (brain)	U73304	0.31 (0.15–0.63)
Translation	PDE4DIP; phosphodiesterase 4D interacting protein (myomegalin)	AI821791	0.27 (0.12–0.59)
	EIF4A2; eukaryotic translation initiation factor 4A, isoform 2	NM_001967	0.38 (0.34–0.43)
Unknown	FLJ22457; hypothetical protein FLJ22457	NM_024901	0.21 (0.13–0.35)
	KIAA1078; KIAA1078 protein	AL110158	0.36 (0.21–0.61)
	CHORDC1; cyteine and histidine-rich domain (CHORD)-containing, zinc-binding protein 1; CHP1	NM_012124	0.37 (0.27–0.50)
	MUM1; melanoma-associated antigen (mutated) 1	NM_032853	0.38 (0.27–0.53)

Shown are all sequences whose expression ratios were 0.40 or less. Expression ratios are shown as geometric means, with 95% confidence intervals in parentheses.

Table 4. *Sequences most strongly increased by moderate hypothermia*

Functional Category	Common Name(s)	GenBank Identifier	Cold-to-Control Expression Ratio
Cell growth, proliferation, and differentiation	CTNNBIP1; catenin, beta interacting protein 1; ICAT	NM_020248	4.2 (2.4–7.2)
	GAS7; growth arrest-specific 7	BE439987	3.0 (2.3–3.8)
Growth factors and related proteins	IGF1; insulin-like growth factor 1 (somatomedin C)	AI972496	2.5 (1.2–5.1)
Immune function	CLEC7A; C-type lectin domain family 7, member A; CLECSF12; C-type (calcium-dependent, carbohydrate-recognition domain) lectin, superfamily member 12; C-type lectin 1	AF313468	3.1 (1.6–6.2)
	EBI2; Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	NM_004951	3.0 (1.5–6.3)
	LSP1; lymphocyte-specific protein 1; pp52; WP34	NM_002339	3.0 (1.7–5.5)
	CD-14; CD14 antigen	NM_000591	2.5 (1.3–5.0)
Membrane transport	SLC15A3; solute carrier 15, member 3; peptide transporter 3	NM_016582	3.2 (2.4–4.1)
Metabolism and biosynthesis	COX11; COX11 homolog, cytochrome *c* oxidase assembly protein (yeast)	BC005895	2.6 (2.3–2.9)
Post-translational processing	B3GALT4; UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4; beta-3 GALT	AB026730	7.3 (4.4–12.2)
Protein degradation	PSMD11; proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	BF432873	5.7 (2.4–13.5)
RNA stability and degradation	CIRBP; cold-inducible RNA-binding protein; CIRP	NM_001280	3.7 (2.9–4.8)
		NM_001280	2.7 (2.2–3.3)
Signal transduction	PPM1F; protein phosphatase 1F (PP2C domain containing); KIAA0015	NM_014634	2.6 (1.8–3.6)
Transcription	MAFF; V-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein F	AL021977	6.0 (3.6–10.2)
Unknown	*Homo sapiens* (clone B3B3E13) Huntington's disease candidate region mRNA fragment	L37198	8.1 (3.8–17.1)
	CDNA FLJ11898 fis, clone HEMBA1007322	AK021960	4.9 (2.5–9.6)
	C6orf62; chromosome 6 open reading frame 62	AW972292	3.6 (2.3–5.8)
	FLJ20716; hypothetical protein FLJ20716	NM_017938	3.3 (1.2–9.0)
	TTLL3; tubulin tyrosine ligase-like family, member 3	AF078842	2.8 (1.6–4.6)
	PSCDBP; pleckstrin homology, Sec7 and coiled-coil domains, binding protein	L06633	2.7 (1.1–6.8)
	DKFZP434C171; DKFZP434C171 protein	AL080169	2.6 (1.6–4.1)
	KLHL7; Kelch-like 7 (*Drosophila*)	NM_018846	2.5 (1.6–4.0)

Shown are all sequences whose expression ratios were 2.5 or more. Expression sequences are shown as geometric means, with 95% confidence intervals in parentheses.

Both CIRBP and RBM3, genes widely known to demonstrate increases in expression at the level of mRNA as a result of hypothermia (4, 13, 14), met our statistical significance and post hoc filter criteria for inclusion as genes affected by hypothermia. Two sequences corresponding to CIRBP were strongly increased by hypothermia, showing geometric mean expression ratios of 3.7- and 2.7-fold (Table 4). Although somewhat less strongly affected by hypothermia, a sequence corresponding to RBM3 also met our inclusion criteria for upregulated genes [geometric mean expression ratio of 2.1 (95% CI: 1.5–3.0)].

Additionally, we specifically examined the effect of moderate hypothermia on the expression of TNF-α and IL-1β, both of which have been found to have hypothermia-augmented expression in THP-1 cells stimulated with LPS (5). TNF-α showed a statistically significant increase in expression [geometric mean expression ratio of 1.9 (95% CI: 1.5–2.3)] and met our post hoc presence/absence call inclusion criterion but fell below the twofold expression ratio required to be included in the final list of genes. IL-1β was not expressed in any of the unstimulated control cells and did not exhibit a significant change in expression as a result of moderate hypothermia. Additionally, when we scanned the list of genes that met our inclusion criteria for cytokines, we found that hypothermia significantly increased the expression of IL-8 [geometric mean expression ratio of 2.1 (95% CI: 1.4–3.0)] and calgranulin A(S100A8) [geometric mean expression ratio of 2.3 (95% CI: 1.0–5.0)].

Confirmatory RT-PCR. We performed RT-PCR on a select number of sequences of interest. Figure 3 shows the results of three additional experiments that were performed to confirm the microarray findings. The genes tested included the immune function proteins lymphocyte-specific protein (LSP)-1, C-type lectin-1, and CD14 (all listed in Table 4); the growth factor IGF-I (Table 4); transcription factor v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family member F (MAFF; Table 4); HSP105 and HSP47 (both listed in Table 3); control sequences cyclophilin A and GAPDH (both listed Table 2); and the RNA-binding protein CIRBP (listed in Table 4). We also examined TNF-α, which met all but our twofold cutoff criterion for inclusion. As illustrated in Fig. 3, in each of the three paired sets of samples, the qualitative RT-PCR confirmed the predictions made by the microarray data regarding the effect of cold exposure on the direction of change in expression. There was, however, noticeable variation in the absolute levels of expression produced by the same stimulus in different experiments (Fig. 3).

DISCUSSION

In summary, THP-1 cells exposed to moderate hypothermia (32°C) for 24 h demonstrate changes in gene expression that are extensive, broader than previously recognized, and include a substantial component of downregulation. Indeed, the number of sequences that demonstrated decreased expression as a result of moderate hypothermia exceeded the number of sequences that demonstrated increased expression and included important categories such as HSPs.

Microarray data are frequently criticized for generating large numbers of false-positive results. If one accepts a *P* value of

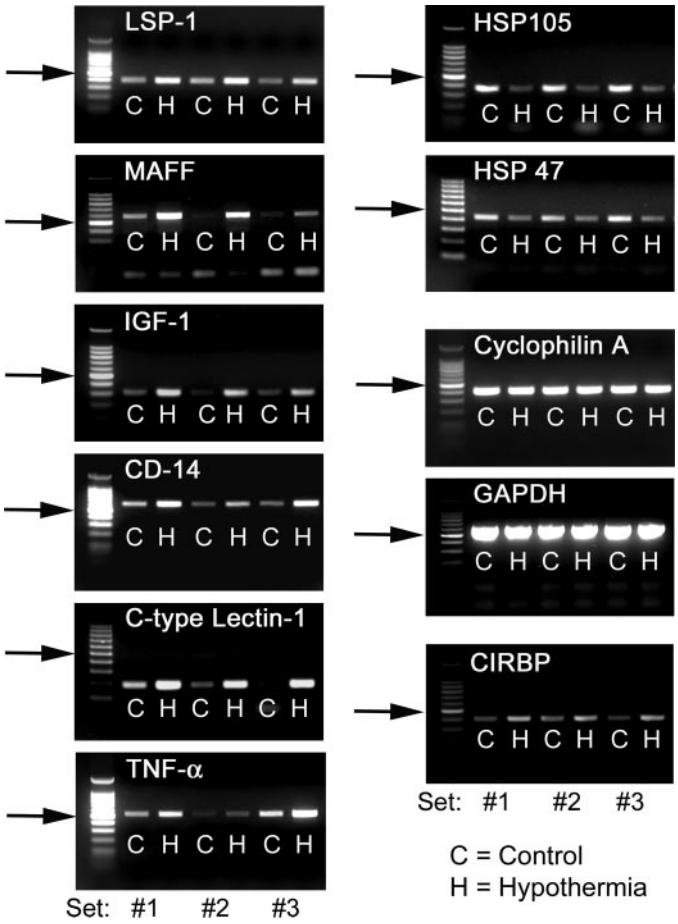

Fig. 3. Confirmatory RT-PCRs. Paired samples obtained from 3 additional experiments are represented. The ladder bands (*far left*, unlabeled lane) occur in 100-bp increments; arrows point to the bright 500-bp bands in the ladders. LSP-1, lymphocyte-specific protein-1; MAFF, v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family member F; HSP105 and HSP47, heat shock 105- and 47-kDa protein, respectively.

$\leq$0.05 as statistically significant, one would expect about 1,100 of the 22,283 sequences on the array to be labeled as showing significant differences of expression by random chance alone, even in the absence of a true difference. The number of false-positive reports can, however, be reduced substantially by a variety of data-filtering techniques (10, 22). The approach used here has been applied previously, with minor variations, to the study of heat shock (20), hypoxia (18), and vitamin D exposure (22) and, in those contexts, yielded results that were both highly congruent with the published literature and replicable by alternative techniques. In the present study, significant changes in expression were observed in sequences corresponding to genes such as CIRBP and RBM3, which are widely accepted as being true "cold shock" genes (19). Conversely, none of the genes we examined as control sequences met our criteria for inclusion as cold responsive, and, in fact, only one (cyclophilin A) showed a statistically significant, although minor, change in expression. Furthermore, after post hoc filtering, the 95% CI on the geometric mean expression ratios were generally well removed from onefold, suggesting that in the final, filtered list of genes reported in Tables 3 and 4, the number of false-positive reports is likely to be small. Finally, we were able to confirm a number of critical findings in separate experiments by RT-PCR; although qualitative in nature and only capable of reliably detecting large changes in expression (at least, substantially greater than the 10% change in expression observed for cyclophilin A on the microarray), the results nevertheless give added confidence that several of the more interesting changes observed in Tables 3 and 4 represent true-positive findings.

A known disadvantage to strict post hoc filtering of microarray data is that, while it enhances the efficiency of discovery of new genes affected by a particular stimulus by reducing the number of false-positive reports, this occurs at the expense of generating false-negative reports, i.e., it excludes sequences that are truly affected by the stimulus in question but that don't meet one of the post hoc filter criteria. This is true as well for the general approach used in this study (22). Indeed, we found that TNF-α was significantly increased both by the microarray data [geometric mean expression ratio of 1.9 (95% CI: 1.6–2.3)] and on confirmatory RT-PCR (Fig. 2) but did not meet one of our post hoc criteria for inclusion (an expression ratio of at least 2-fold). Thus, although the lists of genes in Tables 3 and 4 contain at most a few false-positive reports, they cannot be considered comprehensive.

It is interesting to note that, in this study, moderate hypothermia produced a decrease in the level of expression of a number of HSP mRNA species, including large decreases in the expression of sequences representing physiologically important HSP genes such as HSP70, HSP110, and HSP105 (Table 3). Cold stress has previously been believed to lead to increased expression of HSPs (for a review, see Ref. 19); however, the studies that specifically addressed this issue generally analyzed HSP expression after a period of rewarming rather than during the period of hypothermia itself, as was done here, and often used lower temperatures than those applied here. Indeed, in experiments in which gene expression in HepG2 cells was examined both at the end of a hypothermic exposure at 31°C and again after a period of recovery at 37°C (unpublished data, submitted elsewhere), HSP expression tended to show a pattern of decreased expression during cold stress followed by increased expression after rewarming. It therefore appears that the HSP response to cold depends critically on timing and intensity, with moderate hypothermia itself tending to produce decreases in HSP mRNA expression and rewarming after moderate hypothermia tending to produce a mild heat shock response.

In addition to HSPs, it appears that cold exposure per se can affect the expression of a number of molecules known to be involved in immune and somatic responses, including IL-8, CD14, IGF-I, and TNF-α. Cold exposure modifies the response of monocytic cell lines to stimulation LPS by enhancing NF-κB gene-dependent expression (5). The present data suggest that cold itself may produce changes in the expression of important modulators of the immune response.

The results of this study should be interpreted with several important limitations in mind. First, we studied a cancer cell line, THP-1, as a model for mononuclear phagocytes; the effects of hypothermia on gene expression may therefore differ in important ways from what would be observed in a population of terminally differentiated cells, such as peripheral blood mononuclear cells or THP-1 cells that have been forced to differentiate by means of in vitro manipulations. Second, it is theoretically possible that cold exposure for 24 h led to selec-

tive dropout of certain cell subpopulations This would tend to produce apparent changes in gene expression that are not due to changes in the production or degradation of mRNA species but rather due to changes in the distribution of cell subtypes. However, the use of an immortal cell line model decreased the likelihood of this occurrence, at least in principle. Third, cold exposure might conceivably lead to changes in the state of differentiation of THP-1 cells, which have been found to occur in response to in vitro stimuli other than hypothermia (3, 8). However, we found no significant changes in the expression of sequences corresponding to critical differentiation antigens (specifically, CD68, CD36, and CD11b), nor did we notice any obvious changes in THP-1 cell morphology, cell-cell adhesion, or cell adhesion to artificial surfaces with over 24 h of exposure to 32 vs. 37°C. Fourth, as noted, our post hoc filter criteria were designed to reduce false-positive reports and, as a result, likely excluded a number of sequences that are indeed affected by cold stress. Fifth, our qualitative PCR experiments can only confirm directions of change in gene expression, not magnitude. Additionally, although the comparisons between controls and hypothermic samples all led to consistent observations, there was noticeable variation in the absolute levels of expression observed across experiments. Potential sources of this variation are well established and include both inherent differences in baseline and activated levels of gene expression (a well-known issue with THP-1 cells) and technical issues such as minor differences in RNA loading that are inherent to qualitative PCR techniques. A precise definition of the magnitude of the changes in expression shown in Fig. 2 would require use of quantitative techniques such as real-time PCR. Finally, it is unclear from the examination of a single cell type alone how extensively the findings may be generalized to other cell types. However, about 25 of the genes reported here also demonstrated large responses to hypothermia in the hepatocytes cell line HepG2 (unpublished data). This suggests that the gene expression response to hypothermia includes both responses that are cell type specific and responses that are general.

Although there is hazard in extrapolating findings made in vitro to the complex processes that occur in vivo, it is noteworthy that the cold exposure delivered in this study was roughly comparable with the degree of cold exposure that has been reported to lead to benefit in studies of therapeutic hypothermia (1, 9, 11, 12, 17). These studies could only speculate about the mechanisms by which benefit was produced but did not consider the possibility of cold-induced changes in gene expression. The present findings suggest that the hypothermic exposure used in these studies might have been sufficient to produce a gene expression response. It is therefore plausible that some of the effects of moderate hypothermia on tissues and systemic function might be mediated, at least in part, by cold-induced changes in gene expression of circulating mononuclear cells. Further work will be required to test this hypothesis.

In conclusion, the exposure of THP-1 cells in vitro to moderate hypothermia (32°C) for 24 h led to changes in gene expression that were extensive and included a significant component of downregulation. Moderate hypothermia without rewarming produced a decrease in the expression of a number of HSPs and also affected the expression of a number of molecules capable of modulating immune responses and other somatic effects. Our work suggests that cold responses differ substantively from heat shock responses and raises the question of whether or not changes in gene expression might contribute to some of the systemic effects of prolonged hypothermia.

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DISCLAIMER

The views, opinions, and findings contained in this publication are those of the authors and should not be construed as an official United States Department of the Army position, policy, or decision, unless so designated by other documentation.

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